PRECLINICAL STUDIES ON HERBAL EXTRACT AND PHYTOCHEMICAL AS MONOCYTE CHEMOATTRACTANT PROTEIN-1 (MCP-1) INHIBITOR FOR DIABETIC COMPLICATIONS

SUMMARY FOR THE DEGREE OF

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INTRODUCTION

Diabetes mellitus (DM) comprises a group of metabolic disorders characterized by hyperglycemia, polyuria, weight loss and thirst. DM may arise due to defects in insulin secretion and its action, or both, which alters carbohydrate, fat and protein metabolism in individuals. International Diabetes Federation (IDF) has estimated that diabetes is one of the fastest growing global health crises of the 21st century. In 2019, it was estimated that 463 million people are suffering from diabetes. This number is proposed to reach 578 million by 2030 and 700 million by 2045. Hyperglycemia causes almost 4 million deaths per year. Diabetes affects not only the individual but also has a great impact to affect their families. It has broad socio-economic consequences and may affect national productivity and economies (WHO Library, 2016; IDF Atlas, 2019).

Hyperglycemia-induced oxidative stress augments auto-oxidation of glucose and produces AGEs, and this stimulates polyol pathway (Gugliucci 2017). A rise in oxidative stress in the cell causes a release of various cytokines like interleukins (IL), transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), nicotinamide adenine dinucleotide phosphate oxidase (NOX 1 & 4) and sirtuin 1 (SIRT1).

Oxidative stress and cytokine release inhibit nitric oxide production. These molecular mechanisms diminish endothelial function and capillary vasodilation. Hence, the hypoxic condition is developed in vascular tissues (Sanada, Matsui, and Yasuda 2016). These drastic changes in the microvasculature lead to abnormal signaling and damage the vital organs. MCP-1 acts as a connecting link in several inflammatory pathways impacting progression of diabetic complications. MCP-1 is involved in expression of various pro-inflammatory and inflammatory proteins via chemotactic activity via customized N-terminal residue. Normally, MCP-1 remains in the cytosol in inactive form. Hyperglycemia leads to generation of oxidative stress and inflammation that upregulates the activity of MCP-1 through AGE/ RAGE, MAPK, JAK–STAT, TLR4, AMPK-SIRT1, TNF-α/ JNK, MEK-ERK, p38 MAPK/NF-κB, VEGF and NLRP3 inflammasome pathway. Hyperglycemia increases the expression of MCP-1. This upregulated MCP-1 causes damage to microvasculature of vital organs, which results in diabetic complications. Due to involvement of MCP-1 in progression of diabetic complications, targeting MCP-1 will provide new way for treatment of diabetic complications. Natural and synthetic MCP-1 inhibitors have been studied in management of diabetic complications such as diabetic nephropathy, retinopathy, neuropathy and cardiomyopathy. Hypoglycemics, Renin-angiotensin blockers,

glucagon, insulin, and antihypertensive agents are the standard treatment options for diabetes and associated complications.

However, these therapies are not entirely efficient for the treatment of diabetic complications. Current evidences showed that Monocyte Chemoattractant Protein-1 (MCP-1) has diverse functions and plays a crucial role in inflammation and chronic pain involved in diabetic complications.

From Ancient times herbal extracts and phytoconstituents are used for the treatment and management of various diseased conditions. Herbal extracts and phytoconstituents has created great interest in scientist for treatment and management of diabetes and diabetic complications.

Aloe vera contains more than 75 medicinally important phytoconstituents. Among them, active phytoconstituents are classified as polysaccharides, enzymes, amino acids, vitamins, lignin, saponins, minerals, anthraquinones, steroids, salicylic acids, triglycerides, lectin-like substances, hormones (Shelton, 1991; Surjushe, Vasani and Saple, 2008) and other miscellaneous compounds such as lupeol which is reported as MCP-1 inhibitor (Gangadharan et al. 2019; Wang et al. 2016).

Paeonol is a bioactive phenol extracted from plants like *Paeonia suffruticosa*, *Paeonia lactiflora*, *Dioscorea japonica*, and *Arisaema erubescens*. Paeonol has been reported to have significant effects on various disease conditions like fever, headache, cold, inflammation, skin diseases, neuralgia, allergy, rheumatoid arthritis, etc. (Liang Zhang, Li, and Liu 2019). Paeonol is reported to have MCP-1 inhibitory activity (Ping et al. 2014)

RATIONALE

MCP-1 plays a crucial role in progression of microvascular complications of diabetes. This expedites us to hypothesize that down regulation of MCP-1 will be novel targets in treatment and management of micro vascular complications of diabetes. Scientific data also revealed that increased MCP-1 expressions involved in the activation of diabetic complications and decreased expression of these novel targets may enriched the physiology of kidney, brain and eyes.

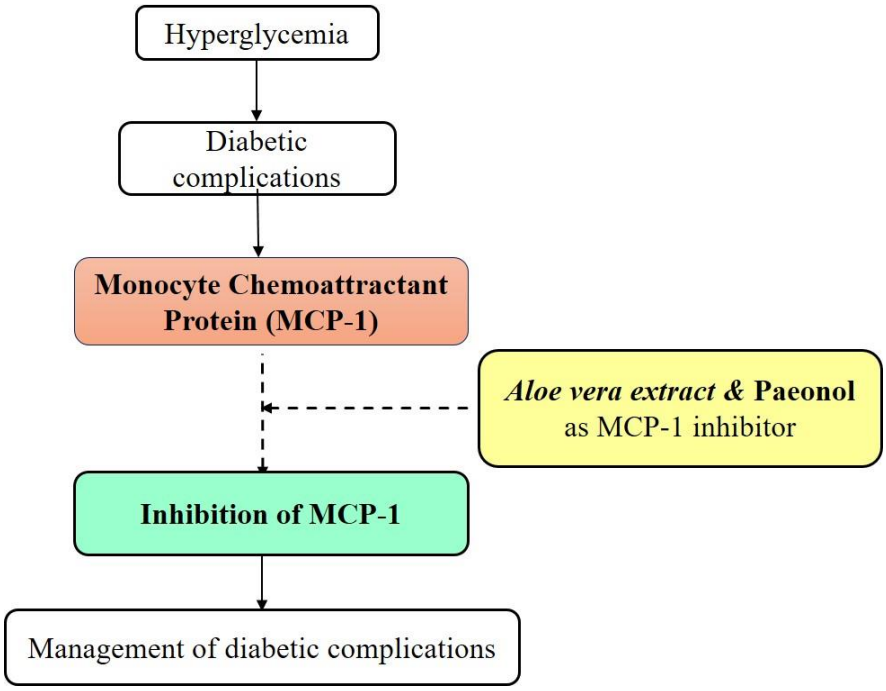

Figure 1: Rationale of the current study

Aloe vera is used as an antioxidant, anti-inflammatory, wound healing, antiulcer/ gastroprotective, antimicrobial, analgesic, cardioprotective, hepatoprotective, nephroprotective, neuroprotective, hypoglycemic and antidiabetic activity, hypotensive and antitumor activity. The *in vitro* and *in vivo* toxicity studies showed that *Aloe vera* extracts could be used at higher dose levels without toxicity. *Aloe vera* showed presence of lupeol, which has been reported for its MCP-1 activity.

Paeonol has been reported to have significant effects on various disease conditions like fever, headache, cold, inflammation, skin diseases, neuralgia, allergy, rheumatoid arthritis, etc. Paeonol has great pharmacological potential as an anti-inflammatory, antidiabetic, neuroprotective, cardioprotective, nephroprotective and anti-tumor agent (Liang Zhang, Li, & Liu, 2019). It has also been reported for MCP-1 inhibitory activity.

Hence, the present study was designed to evaluate the efficacy of lupeol rich *Aloe vera extract* and paeonol in diabetic complications.

OBJECTIVES

1. To study acute and repeated oral dose toxicity of Paeonol

2. To study the effect of *Aloe vera* extract and Paeonol in an experimental model of diabetic nephropathy.
3. To study the effect of *Aloe vera* extract and Paeonol in an experimental model of diabetic retinopathy.
4. To study the effect of *Aloe vera* extract and Paeonol in an experimental model of diabetic neuropathy
5. To study the effect of *Aloe vera* extract and Paeonol in an experimental model of diabetic cardiomyopathy.

MATERIAL AND METHODS

Drugs and chemicals

Paeonol was purchased from Sigma Aldrich (St. Louis, MO, USA). Streptozotocin (STZ) was purchased from MP Biomedicals India Pvt Ltd., India. Diagnostic kits for biochemical analysis were procured from Transasia Biomedicals Ltd, India. Antibodies were procured from Santa Cruz Biotechnology, Inc. and ABclonal Technology.

Procurement and authentication of Aloe vera

Fresh leaves of *Aloe vera* were collected from S. R. Organic, Mumbai in the month of June 2020. The specimen samples were authenticated by scientist from Botanical Survey of India, Government of India, Pune, India.

Extraction and analysis of plant material

Freshly collected leaves of *Aloe vera* were cleaned and dried in hot air oven at 50 ± 5ºC. Dried leaves were powdered and used for the analysis. Diethyl ether extract of *Aloe vera* was prepared by maceration technique. The dried diethyl ether extract was stored in air tight glass container at 2-8°C till the further use. The dried *Aloe vera* extract analysis was done by HPTLC and LC-MS technique.

Experimental animals

Male and Female *Sprague Dawley* rats (6-8 weeks and 180-200 gm) were purchased from the National Institute of Biosciences, Pune, India. All animals were housed in the standard laboratory environment (12 h of light/dark cycle, temperature 22±2˚C and humidity 75±5%) throughout the study. The animal received a standard pellet diet and water *ad libitum* throughout the study.

The animal study protocol was reviewed and approved by the Institutional Animal ethics committee (Approval number- CPCSEA/IAEC/P-80/2018 and P-22/2020). All animal procedures conducted in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, Government of India.

TOXICITY STUDY OF PAEONOL

Acute toxicity study

Acute oral toxicity study of paeonol was carried out as per OECD guideline 423 with minor modifications (Y. A. Kulkarni and Veeranjaneyulu 2012; Y. Kulkarni and Veeranjaneyulu 2010; Test No. 423: Acute Oral toxicity 2002). A single oral dose of paeonol was administered to 03 animals. Paeonol 300, 2000 and 5000 mg/kg body weight dose was selected for acute toxicity. The animal dose was calculated based on their body weight. The animal dose was adjusted weekly to maintain the required dose levels for each rat (i.e., mg/kg/ day). All doses were calculated and administered in such a way that the dose volume does not exceed 1 ml for each animal. The control group received 0.5% CMC (vehicle) at the same dose volume as the treatment group. In acute toxicity study, all animals received dose by the oral route on the first day. All treated animals were observed individually with special attention for toxic signs like diarrhea, convulsions, tremors, salivation, sleep duration and coma for the first 4 h. Morphological observations like change in fur, skin, eyes, respiratory and behavior patterns were done at an interval of 30 min, 1 h, 2 h, 4 h and daily for 14 days after the paeonol treatment. Body weight, feed and water consumption of animals were recorded on alternate days. All animals were weighed on 14th day, sacrificed and gross necropsy was done for any sign of toxicity due to paeonol treatment.

Repeated dose oral toxicity

Repeated dose oral toxicity study of paeonol was carried out as per OECD guideline 407 with minor modifications (Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents 2008). For repeated-dose oral toxicity, four groups were made (n=10). A total of 40 (20 female and 20

male) animals were used in the present study. Each group was containing 05 male and 05 female rats. In repeated dose toxicity study, animals were administered 50, 100 and 200 mg/kg dose by the oral route, daily for 28 days. Treated animals were observed individually for signs like convulsions, tremors, diarrhea, salivation, sleep duration and coma for 28 days. Body weight, feed and water intake of male and female rats of all groups were measured weekly (Shah et al. 2012).

At the end of the study, blood was collected from retro-orbital plexus in heparinized tubes. Hematological parameters were analyzed by using hematology analyzer (Celltac Alpha MEK-6500K, Nihon Kohden, Japan). Biochemical parameters were estimated by using biochemistry analyzer (Erba Chem 7, Germany) (Auti and Kulkarni 2019; Makadiya, Kulkarni, and Apte 2015).Plasma samples were also estimated for potassium, sodium, and chloride levels. All animals were kept in metabolic cages (BIK Industries, India) for urine collection. 24 h of urine was collected from each animal for the estimation of urine output. Fresh urine was centrifuged at 5000 rpm for 10 min and used for the analysis of kidney function parameters such as protein excretion, albumin excretion, creatinine clearance, urea clearance and glomerular filtration rate.

At the end of the study, animals were sacrificed and organ weight of vital organs like brain, heart, lung, liver, kidney, adrenal gland, stomach, intestine, spleen, testes and ovaries was recorded. After weighing, organs were stored in neutral formalin buffer (10%). Vital organs were embedded in paraffin; then, thin sections were taken by using a microtome. Hematoxylins & Eosin (H&E) staining were performed to check the histoarchitecture of all organs.

TO STUDY EFFECT OF *ALOE VERA* EXTRACT AND PAEONOL AS MCP-1 INHIBITOR IN TYPE 1 DIABETIC NEPHROPATHY

Diabetes induction and treatment

Diabetes was induced using Streptozotocin (STZ) (55 mg/kg, *i.p.*). After 7 days of diabetes induction, blood was collected from retro-orbital plexus and plasma separated. Plasma glucose levels were determined using glucose diagnostic kit and Erba biochemical analyser. Animals with plasma glucose level ≥ 250 mg/dL were considered as diabetic and used in the study. After 4 weeks of diabetes induction, body weight of animals were recorded and randomization was done based on body weight and plasma glucose. The *Aloe vera* extract, paeonol, Lisinopril dose was prepared

using 0.5% sodium carboxy methyl cellulose (CMC). Normal control and diabetic control animals were treated orally with 0.5% CMC for next 4 weeks. *Aloe vera extract* and paeonol oral treatment was done for next 4 weeks.

Evaluation parameters

Body weight and plasma glucose

At the end of the study, body weight of individual animal was recorded. Plasma was used for estimation of various biochemical parameters such as glucose, total protein, albumin, creatinine and blood urea nitrogen.

Urine collection, biochemical analysis and kidney function tests

Urine collection (24h) of individual animal was done and urine volume was recorded at the end of the study. Fresh urine sample were used for the estimation of total protein, albumin, creatinine and urea. Kidney function tests were calculated (Mayuresh Sudamrao Garud and Kulkarni 2018).

At the end of the treatment, animals were sacrificed and kidneys were isolated. Ratio of kidney weight to body weight was used as relative organ weight of kidney. One kidney of animal was fixed in 10% neutral buffered formalin (NBF). Another kidney was stored at -80°C and used for oxidative stress analysis.

Estimation of renal oxidative stress

Kidney tissue was crushed properly and subjected to homogenization by using probe homogenizer. Different fractions of the tissue homogenates were prepared as per following method (Noeman, Hamooda, and Baalash 2011). Total tissue homogenate used for estimation of glutathione (GSH) (Ellman 1959) and malonyldihyde (MDA) (Ohkawa, Ohishi, and Yagi 1979) levels. Total homogenate was centrifuged at 2500 g for 20 min at 4˚C to get post-nuclear supernatant and used for the estimation of catalase (CAT) (Luck 1965). Remaining total homogenate was further centrifuged at 10000 g for 20 min at 4 ˚C to get post-mitochondrial supernatant and used for estimation of superoxide dismutase (SOD) (Paoletti, Mocali, and Aldinucci 1990).

Histopathology study of kidney

The kidney tissue embedded in paraffin blocks were sliced into sections with the thickness of 3

µm using microtome. The tissue sections were stained with following staining reagent hematoxylin-Eosin (HE), Masson Trichome and Periodic Acid Schiff (PAS). Morphometric analysis was performed to evaluate the damage occurred in kidney tissue (Mayuresh Sudamrao Garud and Kulkarni 2017). The following parameters were considered during the scoring: deposition of extracellular matrix, presence of inflammatory cells, tubular atrophy and interstitial cell proliferation. The degree of interstitial fibrosis was graded as 0 for absence of fibrosis and cell infiltration, 1 for single focus of lesion, 2 for more than two isolated foci, 3 for greater than five isolated loci, 4 for greater than 10 isolated foci or fibrosis and infiltration of cells (Garud and Kulkarni 2017). The percentage of collagen content was determined in kidney tissue stained with trichome stain using ImageJ1.51a software.

Western blotting

Western blotting of kidney tissue was carried out to study effect of treatment on MCP-1 levels. Western blot experiment was carried out by using Bio-Rad Laboratories, Inc. (California, USA). Kidney tissue was crushed and added ice cold radio-immuno-precipitation assay (RIPA) buffer. Tissues were homogenized and centrifuged at 8000 rpm for 10 min at 4°C. Supernatant was used for protein determination. Protein concentration was determined by the method of Lowry. (Lowry et al. 1951). Tissue lysate was mixed with one part of 5X Laemmli sample buffer (6.25 µl) in 0.5 ml micro centrifuge tube and subjected to denaturation at 95°C for 5 min on dry bath. Denatured sample were centrifuged at 1000 rpm for 1 min and used for loading. Gel containing 15% acrylamide was used for separation of the proteins. 20 µl of sample (40 µg protein) was loaded along with molecular weight markers. Gel was allowed to run for 10 min at 60 V and then 115 min at 80 V). Electrophoretic assembly was carefully sandwiched in ice-cold transfer buffer. The sequence of electrophoretic assembly such as the PVDF was on the side of cathode (+ ve charge) and the gel was on the side of anode (- ve charge). The transfer tank was placed in ice box and transfer was carried out in cold condition at 200 mA for 6 hr. The blot was rinsed in water and stained with Ponceau S solution to confirm the transfer of bands on PVDF membrane. Further PVDF membrane destained using TBS-T (tris buffered saline + tween 20). PVDF membrane was blocked in 5% non-fatty dry milk in TBS-T (blocking buffer) for 75 min on gel rocker. Primary antibody treatment solution of MCP-1(1:1000) or β-actin (1:5000) was prepared in blocking bufferβ -actin was designated as housekeeping protein. The membrane was incubated with primary antibody solution at room temperature for 2 h.rs on gel rocker. After primary antibody treatment,

blots were washed with TBS-T for 2 times, then washed with TBS-T for 3 times. The washing interval was monitored for 10 min. Secondary antibody solution (1:2000) was prepared in non-fatty dry milk in TBS-T. The blots were incubated with secondary antibody solution at room temperature for 1 hr on gel rocker. After secondary antibody treatment, blots were washed with TBS-T for 3 times. The washing interval was monitored for 10 min. Western blots were developed on X-ray film in dark room. Photographs of the X-ray sheets were taken using photographic scanner. Band intensity was determined as measure of MCP-1 expression and the result was expressed as relative expression (MCP-1/ β-actin) normalize to β-actin.

TO STUDY EFFECT OF *ALOE VERA* EXTRACT AND PAEONOL AS MCP-1 INHIBITOR IN TYPE 1 DIABETIC RETINOPATHY

Diabetes induction and treatment

Diabetes was induced using Streptozotocin (STZ) (55 mg/kg, *i.p.*). After 7 days of diabetes induction, blood was collected from retro-orbital plexus and plasma separated. Plasma glucose levels were determined using glucose diagnostic kit and Erba biochemical analyser. Animals with plasma glucose level ≥ 250 mg/dL were considered as diabetic and used in the study. After 4 weeks of diabetes inductions, body weight of animals were recorded and randomization was done based on body weight and plasma glucose. The *Aloe vera* extract, paeonol, Lisinopril dose was prepared using 0.5% sodium carboxy methyl cellulose (CMC). Normal control and diabetic control animals were treated orally with 0.5% CMC for next 4 weeks. *Aloe vera extract* and paeonol oral treatment was done for next 4 weeks.

Evaluation parameters

Body weight

The body weight of individual animal from all treatment group was recorded at the end of the study.

Evaluation of Electroretinography (ERG)

All animals were housed in a dark for 12 h at the end of paeonol treatment. Light source (M Tech, India) was calibrated with the help of Lux meter at 15 cd.s/m^2. The animals were transferred to an experimental darkroom using a light-proof box for ERG recording. From this point, care was taken

to avoid exposure to light. Animals were weighed and anesthetized with an intraperitoneal injection of urethane at dose 1200 mg/kg. Animals were kept on the elevated platform near a light source. The rat ERG recording electrode (LKC Technologies, MD, USA) was used in current study. A ground electrode (needle) placed near the base of the tail and reference electrode (needle) was placed sub-dermally near head region. Pupil was dilated with 0.5% w/v tropicamide ophthalmic solution. Lens electrode was placed on the cornea. Each animal was subjected to flash of light for 0.1 ms and ERG was recorded using Powerlab data acquisition system (AD instruments, Australia) (Bhatt and Addepalli 2010). The experiment started with the 10 trials/step pulse. Trials were taken 20 ms pre-trigger time and 250 ms post-trigger time. ERG was recorded with pulse intensity 15 cd.s/m^2 for each animal.

The a-wave amplitude was measured by calculating the difference in amplitude between time 0 and the lowest point on the trace (on –X-axis). The b-wave amplitude was measured by calculating the difference in amplitude between the lowest point of a-wave and the highest point on the trace (on X-axis). The a-wave and b-wave latency (time-to-peak) was measured by calculating the elapsed time between the bottom of the a-wave and the top of the b-wave, respectively.

Plasma biochemistry parameters

After recording ERG, blood was collected from retro-orbital plexus and plasma was separated.

Glucose and lactate dehydrogenase levels were estimated by using diagnostic kits and Biochemical analyser (Erba Chem 7, Germany). Animals were sacrificed (CO_2 asphyxiation) and eyes were isolated carefully and weighed. One eye preserved in modified Davidson's fixative solution for histopathology study (OECD 2018).

Preparation of lens homogenate and protein estimation

Lens was separated from eyeballs and subjected to homogenization in phosphate buffer (pH 7.4) using a probe homogenizer (Polytron®, France). Homogenates were centrifuged using a cold centrifuge (Eppendorf, Germany) at 2500 rpm. The supernatant was used for the determination of protein concentration and aldose reductase assay. Protein estimation was carried out by using Lowry's method (Lowry et al. 1951).

Lens homogenates were diluted 20 times with millipore water and used for protein estimation. Bovine serum albumin (BSA) was used as standard and millipore water as blank. 10 µl of tissue homogenate and 10 µl alkaline copper sulfate was added to 96 well plates and incubated for 10 min at room temperature. 0.1 ml of Folin's phenol reagent was added, mixed thoroughly and incubated at 37ºC for 10 min. Absorbance was recorded at 640 nm against blank in a microplate spectrophotometer (Epoch 2, BioTek, USA).

Determination of Aldose reductase activity

Aldose reductase assay was carried in the lens supernatant to determine the effect of paeonol on aldose reductase inhibition. The assay was carried out by using 0.067 M phosphate buffer (70 µl), 25×10^{-5} M NADPH (10 µl), 5×10^{-4} M DL-glyceraldehyde (10 µl) and lens supernatant (10 µl). The pH of the final reaction mixture was adjusted to pH 6.2 by using 1M NaOH. DL-glyceraldehyde was used as a substrate. Aldose reductase assay was carried out in 96-well plate. Wells containing all components except DL-glyceraldehyde considered blank. Absorbance was recorded against blank at 340 nm and 60 sec time interval for 3 min using a microplate spectrophotometer (Epoch 2, BioTek, USA). Aldose reductase activity was calculated ΔOD/min/mg protein and expressed as U/mg protein (Patel et al. 2012).

Determination of sorbitol dehydrogenase activity

Sorbitol dehydrogenase assay was carried out according to methods described previously by Lindstad with slight modification. The test was carried out in 96 well plates. To the blank plate added 117.5 µL of 100 mM Triethanolamine Buffer (pH 7.6), 250 µl 1.1 M D-Fructose Solution, 50 µl 12.8 mM β-Nicotinamide Adenine Dinucleotide (Reduced Form) Solution.

All solutions mixed by inversion and monitored the absorbance at 340 nm (until it gives constant absorbance) using a temperature-controlled microplate spectrophotometer (Epoch 2, BioTek, USA) at 25ºC. Then added 50 µL tissue homogenate and mixed immediately.

Absorbance was recorded at 340 nm for 3 min and obtained the absorbance/ minute using the maximum linear rate. 1% w/v BSA solution used as blank instead of homogenate (Lindstad, Hermansen, and McKinley-McKee 1992; Sigma 1998). Sorbitol dehydrogenase activity was calculated and expressed in U/mg protein.

Assessment of oxidative stress parameters

Lens tissue homogenates were prepared. Estimation of total protein was done by Lowry's method (1951). The lens homogenate was used for estimation of GSH and MDA. The post-nuclear and post-mitochondrial supernatant was used for the estimation of CAT and SOD as described earlier.

Relative organ weight and histopathology

Weight of eye to body weight ratio was calculated to get relative organ weight of eye. Eyes preserved in 4% paraformaldehyde, Davidson's fixative solution were used for the histopathology study. 20 µm slices of eye tissue were made by using microtome (Leica Biosystems, Germany) and prepared paraffin blocks (OECD 2018). All sections were stained by Mayer's Hematoxylin & Eosin and determined change in retinal thickness under a digital microscope with 400X magnification (Kjellström et al. 2006).

Western blotting

Expression of MCP-1 in the lens normal control, Aloe vera extract, Paeonol and standard treatment groups were checked using MCP-1 (rabbit; 1:500) (ABclonal Technology, USA) primary antibody and peroxidase-labeled goat anti-rabbit IgG secondary antibody.

TO STUDY EFFECT OF *ALOE VERA* EXTRACT AND PAEONOL AS MCP-1 INHIBITOR IN TYPE 1 DIABETIC NEUROPATHY

Diabetes induction and treatment

Diabetes was induced using Streptozotocin (STZ) (55 mg/kg, *i.p.*). After 7 days of diabetes induction, blood was collected from retro-orbital plexus and plasma separated. Plasma glucose levels were determined using glucose diagnostic kit and Erba biochemical analyser. Animals with plasma glucose level ≥ 250 mg/dL were considered as diabetic and used in the study. After 6 weeks of diabetes inductions, body weight of animals were recorded and randomization was done based on body weight and plasma glucose. The *Aloe vera* extract, paeonol, Lisinopril dose was prepared using 0.5% sodium carboxy methyl cellulose (CMC). Normal control and diabetic control animals were treated orally with 0.5% CMC for next 4 weeks. *Aloe vera extract* and paeonol oral treatment was done for next 4 weeks.

Body weight and plasma glucose

Body weight of animals was recorded weekly. Blood samples were collected from retro-orbital plexus at the end of the study and plasma was separated by centrifugation at 8000 rpm for 15 min.

Thermal nociceptive threshold

At the end of the study, thermal nociceptive threshold was measured by using tail immersion and Eddy's hot plate test.

Tail immersion test

At the end of paeonol treatment experiment animals were acclimatized to restrainer for 30 min. The tail of experimental animal was immersed in hot water (temperature was maintained at 55 ± 0.5°C). Thermal pain response is time taken by animals to remove the tail out of the hot water. The thermal pain response time was noted for each animal. The cut off time for tail to hot water was 15 s avoid tissue damage (Kulkarni, Agarwal, and Garud 2015; Ramabadran et al. 1989).

Eddy's Hot plate test

After 28 days of paeonol treatment animals were placed individually on pre-heated platform at 55 ± 0.5 °C of hot plate (IITC Life Sciences, USA). Animals were carefully observed for thermal pain response like licking, flickering and jumping. The time required for the first response (Flickering, licking of hind paw or jumping) was recorded for each animal. The cut off time was 15 s avoid tissue damage (Eddy and Leimbach 1953; Padgaonkar et al. 2018).

Mechanical pain threshold

Mechanical pain threshold was measured at the end of the paeonol treatment, by using Randall-Selitto and Von Frey filament method.

Mechanical hyperalgesia

Mechanical hyperalgesia was determined by Randall-Selitto test to determine mechanical nociceptive threshold. This method was used to measure the maximum pain threshold caused due to constantly increasing pressure. All treated animals were immobilized in cloth slings before

starting of experiment. The stimulus was applied at the center of dorsal surface of hind paw. Animals subjected to constantly increasing pressure stimulus using pressure applicator (IITC Life Sciences, USA). The maximum pressure was recorded at which animal shows pain responses like struggling, squeaking and paw withdrawal was recorded with digital meter (IITC Life Sciences, USA). The maximum cut-off pressure was adjusted to 400 g to avoid the tissue damage. Each reading was recorded in triplicate with the interval of 20 min. The mean threshold was considered in final readings (Al-Rejaie et al. 2015; Randall L O and Selitto J J 1957).

Mechanical allodynia

Mechanical allodynia was recorded by using Von Frey filament to measure the nociceptive threshold to a constantly increasing pressure stimuli. Mechanical allodynia is a painful impression caused by innocuous stimuli like pressure. Animals were adopted for min 20 min in elevated cage with wire mesh bottom. Stimulus was applied on the ventral surface of hind paw with Von Frey pressure applicator attached with filament (IITC Life Science, USA). The pressure at which animal shows responses like brisk lifting and paw withdrawal was documented with digital meter (IITC Life Science, USA). The cut-off pressure was set to 400 g to avoid tissue damage. Readings were recorded in triplicate with 20 min interval for each reading and mean values were considered as final readings (Levin, Pearsall, and Ruderman 1978; Romanovsky et al. 2010).

Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV)

Motor and sensory nerve conduction velocity was recorded near sciatica-posterior tibial conducting system of animal. At the end of the study animals were anaesthetized using Urethane (1200 mg/kg i.p.). The body temperature of individual animal was maintained at 37 ± 0.5 °C with homoeothermic blanket and flexible probe (Harvard Apparatus, USA) during the assessment of nerve conduction velocity. The sciatic nerve of the experimental animal was stimulated with single stimulus of 5 V using needle electrodes of 24 gauge at the sciatic notch and tibial nerve (near ankle region). The receipt electrodes were placed in the foot muscles. The H-wave and M-wave reflexes were recorded digitally using data acquisition system (AD Instruments, Australia). The distance between 2 stimulation points were measured manually. The M-wave latencies and H-wave latencies were used for the calculation of MNCV and SNCV, respectively (Ali et al. 2014; Yerra et al. 2018).

Assessment of oxidative stress parameters of nerve

Sciatic nerve tissue homogenates were prepared. Estimation of total protein was done by Lowry's method (1951). Sciatic nerve homogenate was used for estimation of GSH and MDA. The post-nuclear and post-mitochondrial supernatant was used for the estimation of CAT and SOD as described earlier.

Histopathology of sciatic nerve

Sciatic nerve samples were fixed in neutral buffered formalin. Sciatic nerve tissue was embedded in paraffin wax. Paraffin wax embedded tissue blocks were sliced with the Rotary Microtome to get (4-5 µm thickness). Sections of sciatic nerve sections were stained with Hematoxylin & Eosin (H & E) stain. The prepared slides were examined under microscope. Severity of the observed lesions were recorded as NAD =No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4) and distribution was done as focal, multifocal and diffuse.

Neuronal count was performed using Olympus MagVision software (calibrated with micrometer). Initially, image of all the sciatic nerve samples were captured at 400X magnification and images were used for neuronal count (number of neurons/1000mm2) [34].

Western blotting

Western blotting of sciatic nerve was carried out to study effect of paeonol treatment on MCP-1 protein expression. Western blotting was done as method described by Garud (Mayuresh S. Garud and Kulkarni 2017), Chiadak and group (Chiadak et al. 2016) and method described earlier.

TO STUDY EFFECT OF *ALOE VERA* EXTRACT AND PAEONOL AS MCP-1 INHIBITOR IN TYPE 1 DIABETIC CARDIOMYOPATHY

Diabetes induction and treatment

Diabetes was induced using Streptozotocin (STZ) (55 mg/kg, *i.p.*). After 7 days of diabetes induction, blood was collected from retro-orbital plexus and plasma separated. Plasma glucose levels were determined using glucose diagnostic kit and Erba biochemical analyser. Animals with plasma glucose level ≥ 250 mg/dL were considered as diabetic and used in the study. After 6 weeks of diabetes inductions, body weight of animals were recorded and randomization was done based on body weight and plasma glucose. The *Aloe vera* extract, paeonol, Lisinopril dose was

prepared using 0.5% sodium carboxy methyl cellulose (CMC). Normal control and diabetic control animals were treated orally with 0.5% CMC for next 4 weeks. *Aloe vera extract* and paeonol oral treatment was done for next 4 weeks.

Evaluation parameters

Body weight

Body weight of individual animals was recorded at the end of the study.

Plasma biochemical analysis

At the end of study blood samples were collected from retro-orbital plexus and plasma was separated by centrifugation at 8000 rpm for 15 min.

Biochemical parameters such as plasma glucose was estimated as discussed earlier. Plasma triglyceride (TG), total cholesterol (TC), low density lipoprotein- cholesterol (LDL-c), high density lipoprotein-cholesterol (HDL-c), creatinine kinase (CK-M), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were estimated using commercially available diagnostic kits as per manufacturers protocol and biochemical analyser (Erba Chem-7, Germany).

Relative organ weight

After recording of ECG and LVEDP animals were sacrificed by CO_2 anaphylaxis and isolated heart. Weight of individual heart was recorded. Ratio of heart weight to body weight was calculated.

Electrocardiographic analysis

At the end of treatment, ECG was recorded under urethane anaesthesia (1200 mg/kg, *i.p.*) and needle electrode (+ve, -ve and neutral) using (Powerlab, AD Instruments, Australia). The change in PR interval, QT interval, ST segment, QRS duration was determined by ECG.

Haemodynamic parameters

After ECG, the neck of the animal was opened with a ventral midline incision and the left carotid artery was cannulated for the measurement of heart rate (HR), left ventricular end diastolic pressure (LVEDP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean

arterial blood pressure (MABP). The cannula was filled with heparinised saline and connected to a pressure transducer. The haemodynamic parameters were recorded on an powerlab data acquisition system having Labchart software (Wang, Li, and Li 2015).

Estimation of cardiac oxidative stress parameters

Heart homogenate (10 %) was prepared in a 50 mM phosphate buffer, pH 7.4. MDA was measured as thiobarbituric acid reacting substances (TBARS), superoxide dismutase (SOD), Catalase and reduced glutathione was determined according to reported methods described earlier.

Histopathological studies

Rats were sacrificed and circulating blood was removed by cardiac perfusion with 0.1M PBS (pH 7.4; 20–50 ml). After clearance of circulating blood, 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) was perfused for another 5 min (100–200 ml of fixative) to fix the tissues. Heart was then removed from the animal, decapsulated, sliced transversely, and paraffin embedded for light microscopic evaluation. Histopathological changes in heart structure was assessed with help of H & E and Trichome staining.

Western blot analysis of heart tissue

Western blotting of cardiac tissue was carried out by using Bio-Rad Laboratories, Inc. (California, USA) electrophoresis and blotting apparatus as per previously described methods.

RESULTS

Toxicity study of paeonol

Paeonol treated animals showed normal behavior during the first 1 h to 4 h and for the remaining study period. Animals showed no change in fur, eyes and nose. No significant changes were observed in body weight, food and water intake. The single oral dose of paeonol did not show any signs of toxicity at a dose of 300, 2000 and 5000 mg/kg in female rats. No pathological changes were observed in gross necropsy. No abnormal signs in the behavioral pattern were observed in paeonol treated animals. Repeated oral administration of paeonol for 28 days showed no significant change in the body weight of male and female animals when compared to control animals. Food and water intake of all animals in treatment groups was similar to normal control

animals. Hematological parameters did not show statistically significant differences in paeonol treated animals when compared to the control group The kidney function parameter did not show statistically significant differences in paeonol treated animals when compared to the control group. Organs such as the brain, heart, lung, liver, kidney, stomach, intestine, spleen, adrenal glands, testes and ovaries did not show a significant difference in weight in the paeonol treated group compared with control animals. The histopathological study of vital organs did not show any toxicity signs. Maximum tolerated dose of paeonol was found to be 5000 mg/kg in acute dose oral toxicity study. Paeonol was found to be safe at50, 100 and 200 mg/kg dose in repeated dose toxicity study.

Effect of *Aloe vera* extract and paeonol in diabetic nephropathy in type 1 diabetic rats

Aloe vera extract and paeonol oral treatment for 28 days resulted in significant increase in body weight and decrease in relative organ weight of the kidney. The treatment also showed significant decrease in plasma glucose, creatinine, BUN levels and increase in albumin, total protein. *Aloe vera* extract and paeonol treatment showed a significant decrease in urine output, protein and albumin excretion and increase in creatinine, urine urea clearance and glomerular filtration rate. *Aloe vera* extract and paeonol treatment reduced oxidative stress of kidney via increasing the levels of GSH, SOD, CAT and decreasing the levels of MDA. *Aloe vera* extract and paeonol treatment protected kidney from hyperglycemia-induced oxidative stress via reducing mesangial matrix index, basement membrane thickening and mesangial matrix deposition, tubulointerstitial fibrosis index and % collagen content in the kidney. Western blot study indicated that MCP-1 expressions were significantly downregulated after *Aloe vera* extract and paeonol treatment in diabetic rats.

Effect of *Aloe vera* extract and paeonol in diabetic retinopathy in type 1 diabetic rats

Aloe vera and paeonol treatment in diabetic animals showed significant increase in body weight and improvement in ERG via increasing a-wave and b-wave amplitude; and decreasing a-wave and b-wave latency at 15 cd.s/m^2. *Aloe vera* and paeonol treatment significantly decreased plasma glucose levels in diabetic rats. *Aloe vera* extract and paeonol treatment significantly decreased plasma lactate dehydrogenase, aldose reductase and sorbitol dehydrogenase enzyme levels. *Aloe vera* extract and paeonol treatment reduced oxidative stress of eyeball via increasing the levels of GSH, SOD, CAT and increasing the levels of MDA in diabetic rats. The treatment also showed significant increase in relative organ weight of eyeball. The histopathology study of retina of *Aloe*

vera extract and paeonol treated animals showed a significant decrease in retinal thickness in diabetic rats. MCP-1 expressions were not observed in retinal samples in western blotting study.

Effect of Aloe vera extract and paeonol in diabetic neuropathy in type 1 diabetic rats

Aloe vera extract and paeonol treatment significantly increased body weight and decreased plasma glucose levels. *Aloe vera* extract and paeonol treatment showed significant increase in tail withdrawal response in tail immersion test and thermal pain response in Eddy's Hot plate test. *Aloe vera* extract and paeonol treatment in diabetic animals significantly increased in paw withdrawal pressure in Randall Selitto test and paw withdrawal pressure in Von Frey test. *Aloe vera* extract and paeonol treated diabetic animals showed significant increase in motor and sensory nerve conduction velocities. *Aloe vera* extract and paeonol treatment in diabetic animals has reduced oxidative stress in sciatic nerves via increasing the levels of GSH, SOD, CAT and decreasing the levels of MDA. *Aloe vera* extract and paeonol treatment showed increase in neuronal count of sciatic nerve in histopathology study. Western blot study indicated that *Aloe vera* extract and paeonol treatment downregulated MCP-1 expression of sciatic nerve.

Effect of Aloe vera extract and paeonol in diabetic cardiomyopathy in type 1 diabetic rats

Aloe vera extract and paeonol oral treatment improved body weight and lowered plasma glucose levels of of diabetic animals. Electrocardiogram analysis of *Aloe vera* extract and paeonol treatment showed significant increase in PR, QT intervals, ST segment and QRS duration. Haemodynamic analysis showed significant increase in systolic, diastolic, mean arterial blood pressure and heart rate. The ventricular parameters of *Aloe vera* extract and paeonol treated animals showed decrease in LVEDP and decreased +dp/dt and -dp/dt significantly. Plasma biochemical analysis of *Aloe vera* extract and paeonol treated animals showed significant decrease in lipid (TC and TG) and cardiac (LDH, AST, CK-MB) markers. *Aloe vera* extract and paeonol treatment also lowered the cardiac hypertrophy. Treatment also reduced oxidative stress of cardiac tissue via increasing the levels of GSH, SOD, CAT and reducing the levels of MDA. Histopathology study of heart tissue of *Aloe vera* extract and paeonol treated aniamls showed cardioprotective effects by reducing cardiac tissue necrosis, fibrosis, cytoplasmic vacuolation, lymphocytic infiltration in myocardium and % collagen deposition. Western blot study indicated that *Aloe vera* extract and paeonol treatment downregulated MCP-1 expression of cardiac tissue.

CONCLUSION

Aloe vera extract and paeonol treatment has protected kidney, eyeball, heart and sciatic nerve from hyperglycemia-induced oxidative stress in type 1 diabetic rats. *Aloe vera* extract and paeonol treatment will be helpful for the management of diabetic nephropathy, neuropathy, retinopathy and cardiomyopathy via reducing the oxidative stress and inhibiting of MCP-1.

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RESEARCH OUTCOME

Publications

Sr. No.	**Details of publications**	**Impact factor**	**Status**
1.	**Adki, K. M.,** & Kulkarni, Y. A. (2020). Chemistry, pharmacokinetics, pharmacology and recent novel drug delivery systems of paeonol. **Life sciences,** 250, 117544.	3.647	Published
2.	**Adki, K. M.,** & Kulkarni, Y. A. (2020). Potential biomarkers in diabetic retinopathy. **Current Diabetes Reviews,** 16(9), 971-983.	-	Published
3.	**Adki, K. M.,** Murugesan, S., & Kulkarni, Y. A. (2020). In silico and in vivo toxicological evaluation of paeonol. **Chemistry & Biodiversity,** 17(10), e2000422.	2.039	Published

4.	**Adki, K. M.,** & Kulkarni, Y. A. (2021). Neuroprotective effect of paeonol in streptozotocin-induced diabetes in rats. **Life Sciences,** 271, 119202.	3.647	Published
5.	**Adki, K. M.,** & Kulkarni, Y. A. (2022). Paeonol attenuates retinopathy in streptozotocin-induced diabetes in rats by regulating the oxidative stress and polyol pathway. **Frontiers in Pharmacology,** 13, 830103	5.988	Published
6.	**Adki, K. M.,** & Kulkarni, Y. A. (2023). Biomarkers in diabetic neuropathy. **Archives of Physiology and Biochemistry,** 1-16. (Article is in press)	4.076	Published

Book Chapters

Sr. No	**Authors**	**Title of Book**	**Title of the chapter**	**Publisher**
1	**Kaveri M. Adki,** Ankit P. Laddha, AB Gaikwad, YA Kulkarni	Nuts and Seeds in Health and Disease prevention (Second edition)	Potential Role of Seeds From India in diabetes	Elsevier, USA
2	**Kaveri M. Adki,** Ankit P. Laddha, Manisha J. Oza, AB. Gaikwad and YA. Kulkarni	Phytotherapy in the Management of Diabetes and Hypertension, Volume- 3	Terpenoids in Management of Diabetes & Cardiovascular diseases	Bentham Science, Singapore

Sr. No	Authors	Title of Book	Title of the chapter	Publisher
1	**Kaveri M. Adki,** Ankit P. Laddha, AB Gaikwad, YA Kulkarni	Nuts and Seeds in Health and Disease prevention (Second edition)	Potential Role of Seeds From India in diabetes	Elsevier, USA
3	**Kaveri M. Adki** & YA Kulkarni	Structure and Health Effects of Natural Products on Diabetes Mellitus	Glycosides from Natural Sources in the Treatment of Diabetes Mellitus.	Springer, Singapore.
4	Ankit P. Laddha, **Kaveri M. Adki,** AB Gaikwad, YA Kulkarni	Nuts and Seeds in Health and Disease prevention (Second edition)	Beneficial Effects of Nuts From India in Cardiovascular Disorders	Elsevier, USA
5	Ankit P. Laddha, **Kaveri M. Adki,** Manisha J. Oza, AB Gaikwad, YA. Kulkarni	Phytotherapy in the Management of Diabetes and Hypertension, Volume- 4	Medicinal plants from Genus Costus in Management of Diabetes	Bentham Science, Singapore

Presentation:

Kaveri M. Adki and Yogesh A. Kulkarni "Monocyte Chemoattractant Protein-1 (MCP-1) and Diabetic Complications" at **International Diabetes Federation, India** (25^{th} -27^{th} October 2018) Diabetes Complications Congress 2018, Hyderabad.

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